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Original Article

Evaluation of the probiotic and postbiotic potential of lactic acid bacteria from artisanal dairy products against pathogens

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Abstract

Introduction: Probiotic and postbiotic potential of thirty-two strains of lactic acid bacteria (LAB), obtained earlier from artisanal dairy sources in Pakistan, have been investigated against major multi-drug resistant (MDR) and food borne pathogenic bacteria.

Methodology: LAB strains were identified by 16S rRNA gene sequencing and their antibacterial activity was assessed by the microdilution method. Four LAB isolates, *Weissella confusa* PL6, *Enterococcus faecium* PL7, and *Lactobacillus delbrueckii* PL11 and PL13 were shortlisted. Their ability to degrade lactose and safety for human consumption in terms of hemolysis and antibiotic susceptibility were assessed *in vitro*. The antibacterial components in the cell-free supernatants (CFSs) of isolate cultures were characterized biochemically by HPLC.

Results: Acid neutralization but not protease treatment abolished the antibacterial activity of CFSs. Lactic, acetic and propionic acids were the main acids in the CFSs, and acid production peaked in the stationary phase of growth. The antibacterial activity of the LAB cultures resulted from secretion of organic acids that lowered the pH. The strains exhibited variable ability to degrade lactose and were non-hemolytic and susceptible to the most common antibiotics.

Conclusions: These LAB strains are probiotic candidates for further investigation of their postbiotic role in naturally preserving processed foods and for attenuation of lactose intolerance.

Key words: antagonism; multidrug resistance; organic acids; lactose intolerance; lactic acid bacteria; probiotics..

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Introduction

The recurrence of bacterial infections because of the development of antibiotic resistance has become a paramount public health concern [1]. For many years, conventional antibiotics have been at the forefront of infection treatments [2], but due to the rise in bacterial multidrug resistance (MDR), the standard antibiotic regimens have lost effectiveness [3,4]. These MDR pathogens are not only a cause of public concern in hospitals but lately, foods of animal origin have also become a conduit for their spread in communities [5]. In many MDR cases, only a few antibiotics remain effective and it is only a matter of time before these drugs also lose their potency [4]. *Salmonella* spp. is a globally recognized foodborne pathogen. The MDR phenotype has shown a high prevalence in Pakistan [6], but the incidence of extensively drug-resistant (XDR) *Salmonella* spp. has also been reported [7]. *Pseudomonas aeruginosa* is another well-known pathogen implicated in 10% of all hospital-acquired infections the world over [8]. It was found to have MDR, XDR and even pan drug-resistant (PDR) phenotypes in Pakistan [9]. *Staphylococcus aureus* is also regarded as a significant human pathogen frequently occurring in food of animal origin [10] and increasingly reported to be of the MDR variety [11]. Usually a harmless member of the gut microbiome, *Escherichia coli* can also be an opportunistic pathogen causing recalcitrant urinary tract and bloodstream infections [12,13]. Lately, its strains too have been appearing in Pakistan with purported MDR characteristics [14]. After weaning, around 75% of the world's population experiences a decline in lactase activity in the small intestine due to genetic factors [15]. Low level of lactase activity cause maldigestion of lactose and malabsorption in the small intestine [16]. Lactose maldigestion affects around 60% of Pakistan's adult population [17]. Treatment of lactose intolerance is currently restricted to medications and supplements that either have a preventive or a symptom management

function [18]. Lactose-free products and non-dairy fermented foods are also available in markets [19], but lactose is important especially for children, as it is the only disaccharide that does not appear to accentuate the risk of dental caries [20].

To replace the steadily decreasing arsenal of effective antimicrobials and to alleviate lactose intolerance, several alternatives are being tested. Foremost among these strategies are metabolites produced by LABs either during their lifetime or after death, the latter known as postbiotics [21]. Because of their broad-acting antibacterial activity and unique mechanisms of action, these products hold much promise against human pathogens [22] and are being increasingly applied in clinical and industrial settings. Several different substances, such as organic acids (lactic, phenyl lactic and acetic acid), ammonia, free fatty acids, acetaldehyde, diacetyl, hydrogen peroxide, and even peptides have been identified as the underlying antimicrobial agents [23]. Candidate bacterial strains or their postbiotics could be screened as options for treating MDR infections or eliminating pathogens from the food chain. A collection of LAB gathered from dairy foods were identified and characterized for their antibacterial activity against MDR and pathogenic bacteria and capacity to relieve lactose intolerance.

Methodology

LAB strains and culture conditions

The thirty-two LAB isolates were obtained from the culture collection of the National Probiotic Lab, National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan (Table 1) and were regrown on de Man, Rogosa and Sharpe (MRS, Merck, Darmstadt, Germany) agar plates with incubation for 24 hours at 37˚C. The identities of the partial 16S rRNA gene sequences of the isolates previously submitted to GenBank were confirmed by a BLAST database search on NCBI. Sequences with ≥

Table 1. Detail of LAB strains used in present study.

Strain code*	Source	Accession number	Strain identification
PL1	Raw cow milk	KT626385	*Lactobacillus plantarum*
PL3	Homemade yogurt	KT626387	*Lactobacillus rhamnosus*[1]
PL4	Homemade yogurt	KT626388	*Lactobacillus delbrueckii*
PL5	Raw cow milk	KT626389	*Lactobacillus Paracasei*[1]
PL6	Raw cow milk	KT626390	*Weissella confusa*
PL7	Raw cow milk	KT626391	*Enterococcus faecium*
PL8	Raw cow milk	KT626392	*Enterococcus faecium*
PL9	Homemade yogurt	KT626392	*Lactobacillus delbrueckii*
PL10	Raw cow milk	KT626394	*Weissella paramesenteroides*
PL11	Homemade yogurt	KT626395	*Lactobacillus delbrueckii*
PL12	Homemade yogurt	KT626396	*Lactobacillus delbrueckii*
PL13	Homemade yogurt	KT626397	*Lactobacillus delbrueckii*
PL14	Raw cow milk	KT626398	*Lactobacillus sakei* subsp. *sakei*
PL15	Raw cow milk	KT626399	*Lactobacillus sakei* subsp. *sakei*
PL16	Local cheese	KT626400	*Streptococcus lutetiensis*
PL17	Local cheese	KT626401	*Enterococcus faecium*
PL18	Local cheese	KT626402	*Lactobacillus paracasei*[1]
PL19	Local cheese	KT626403	*Leuconostoc lactis*
PL20	Local cheese	KT626404	*Lactobacillus fermentum*[1]
PL21	Raw goat milk	KT626405	*Weissella confusa*
PL22	Raw sheep milk	KT626406	*Lactobacillus fermentum*
PL23	Raw buffalo milk	KT626407	*Streptococcus lutetiensis*
PL24	Raw cow milk	KT626408	*Lactobacillus fermentum*[1]
PL25	Raw goat milk	KT626409	*Lactobacillus fermentum*[1]
PL26	Raw goat milk	KT626410	*Lactobacillus fermentum*[1]
PL27	Raw goat milk	KT626411	*Lactobacillus fermentum*[1]
PL28	Raw sheep milk	KT626412	*Lactobacillus fermentum*[1]
PL29	Raw cow milk	KT626413	*Lactobacillus fermentum*[1]
PL30	Raw sheep milk	KT626414	*Lactobacillus fermentum*[1]
PL31	Raw cow milk	KT626415	*Lactobacillus fermentum*[1]
PL32	Raw cow milk	KT626416	*Lactobacillus fermentum*[1]
PL33	Raw cow milk	KT626417	*Lactobacillus fermentum*[1]

***** stocks from NPL culture collection[1] isolation first reported in an earlier study [67].

99% similarity to the previously published sequences were used as the criteria to confirm species identity. The sequences were aligned using the CLUSTAL W program of the software MEGA 7.0.2. A phylogenetic tree was created using forty-five 16S rDNA sequences comprising the thirty-two sequences of LAB used in this study. Thirteen sequences were obtained from GenBank that were firmly related to the different species obtained in this study. The sequence of *Alkalibacterium olivapovliticus* (AB294175) was also recorded and used as an outgroup.

Indicator strains and culture conditions

The pathogenic bacterial strains used in this study were obtained from the Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. *Staphylococcus aureus* N1, *Escherichia coli* SS1, and *Salmonella enterica* ser Typhi D1 were previously isolated from infected patients and all exhibit an MDR phenotype [24-26]. A non-MDR strain of *Pseudomonas aeruginosa* ATCC 9027 was chosen because it is regarded as a reference strain for evaluating antimicrobial effectiveness in standardized testing protocols worldwide (ATCC® MP16™). All of these indicator strains were cultivated for 18-24 hours in nutrient broth (Merck®, Darmstedt, Germany) at 37˚C with shaking at 200 rpm.

Cell-free culture supernatant preparation

The LAB strains were cultured in enriched media (MRS broth) at 37˚C for 16 hours and sub-cultured in fresh media of the same type until the stationary phase was attained (Figure 1). The cell-free supernatants (CFS) were obtained by centrifugation of the LAB cultures at 4500 ×g for 20 minutes at 4˚C and filter sterilized by passing through a syringe filter (FilterBio®, Nantong, China) of 0.22 µm pore size.

Antibacterial activity of CFS by microdilution method

Overnight cultures of pathogenic bacteria were inoculated into fresh liquid medium (1:100 dilution in nutrient broth) and grown to an OD_{630} of 0.1. For finding out the minimum percentage of CFS that inhibits the growth of target pathogens, the pathogenic cultures were incubated without CFS (control) or with concentrations of CFS ranging from 1 to 18% (v/v). Aliquots of 200 µL from each culture were immediately transferred to a 96-well microtiter plate (Nunc®, Roskilde, Denmark), which was incubated in a microtiter plate reader (Spectramax 384 PLUS®, Sunnyvale, CA, USA) at 37˚C for 16 hours. The growth of the pathogenic bacteria was monitored by measuring absorbence at 630 nm every 15 min. The antimicrobial activity was expressed as percent inhibition of pathogen growth relative to controls grown without CFS. All experiments were performed twice with readings taken in triplicate. The MIP (minimal inhibitory percentage) of CFS is the lowest percentage of CFS that resulted in ≥ 95 % reduction in growth and was determined by subtracting the CFS/culture OD_{630} from that of the cell-free medium [27].

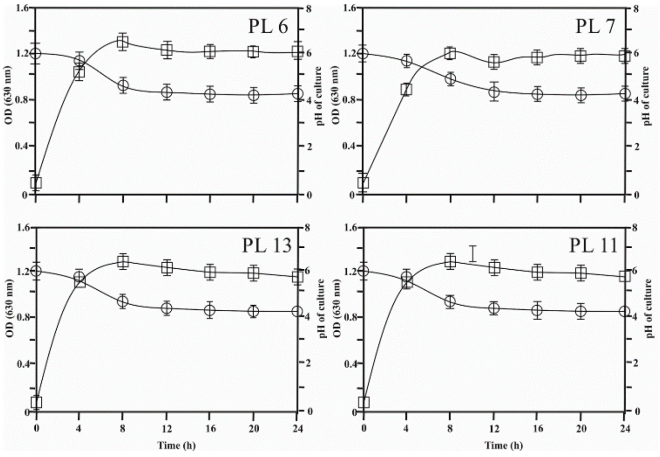
Agar well diffusion assay

The capacity of the shortlisted isolates to inhibit MDR pathogenic bacteria was also examined using indicator organisms in an agar well diffusion assay [28]. Twenty-five mL of liquefied 0.8% (w/v) nutrient agar was mixed with an active overnight culture of indicator isolate (1%, v/v), poured into sterile petri dishes and allowed to solidify. Wells were made with a sterile 8 mm diameter borer and filled with CFS from the LAB isolates. The dishes were incubated for 2 hours at 4°C to permit CFS diffusion into the agar, and then all the plates were incubated at 37°C for 24 hours. The diameter (mm) of the inhibition zone was a measure of the extent of antimicrobial activity. The assays were carried out three times independently.

Nature of the antibacterial metabolite

To investigate the chemical nature of the potentially inhibitory substances secreted by *Weissella confusa* PL6, *Enterococcus faecium* PL7, *Lactobacillus delbrueckii* PL11, and PL13 strains, the CFSs were subjected to a variety of tests. To determine if

Figure 1. Reduction in pH of the culture media during 24 hours growth of select LAB strains.



Data are the mean ± SD of at least three independent experiments. (Squares indicates the growth; circles indicates pH).

surfactants were produced by the test strains, a drop-collapse test was carried out as described [29]. Briefly, 100 µL aliquots of CFS were added to wells of sterile 96-well microtiter plates (Nunc®, Roskilde, Denmark) and 5 µL of crude motor oil was pipetted on top of each. A result was interpreted as positive for biosurfactant when the drop diameter was at least a millimeter larger than that produced by pipetting oil onto distilled water (negative control). Three readings were done for each test. To determine if organic acids were the potentially inhibitory substance, the CFSs were neutralized to pH 6.5-7.0 using 2M NaOH and compared to untreated CFSs. To test whether the inhibitory substances were proteins, the CFSs wee treated with 600 mAU (Anson units) per mL of proteinase K (Merck, Darmstadt, Germany) and 250 µg/mL trypsin (Merck, Darmstadt, Germany). To check heat sensitivity, the CFSs were incubated at 80°C to 95°C for 60 minutes and 121°C for 15 minutes. The treated CFSs (8%, v/v) and untreated controls were then checked for antibacterial activity as described above. All the experiments were performed in triplicate.

Percent inhibition = (a-b)/a×100, where a = OD_{630} of control (pathogen grown under optimal conditions, without CFS) and b = OD_{630} of the CFS-treated group.

Organic acid identification and quantification

To determine the stage at which the LAB strains produced the most acid and lowest pH, cultures were grown in MRS medium for 24 hours at 37°C and 2 mL samples were taken hourly for pH measurements of the supernatants . After 14 hours of growth, CFSs were obtained, filter-sterilized (0.22 µm, FilterBio®, Nantong, China) and analyzed by HPLC. The organic acids were identified and quantified using an Agilent 1200 series HPLC instrument. The stationary phase was the Agilent Hi-Plex H column (300 × 6.5 mm), which was used with the Agilent PL-Hi-Plex H Column (50 × 7.7 mm) maintained at a temperature of 65°C. Five millimolar sulfuric acid was used as the mobile phase with an isocratic flow of 0.6 mL per min. External standards (lactic acid, acetic acid, propionic acid, oxalic acid, formic acid, malic acid, and succinic acid) were run to identify and quantify the organic acids present in CFS. Both the UV spectra and retention times were used to confirm the identity of specific analytes.

Acidic pH tolerance

The acid tolerance assay was performed according to a published protocol [30] with modifications. Overnight-grown LAB strains were inoculated into sterile PBS at a pH of 3 and 7 (control) and incubated aerobically for 3 hours at 37°C. Following incubation, tenfold serial dilutions (up to 10^{-6}) of each bacterial isolate were prepared using PBS, and 50 µL of 10^{-3} to 10^{-6} dilutions were spread on MRS agar and incubated aerobically at 37°C for 24 h. After incubation, colonies on the plates were counted using a colony counter (QCount®, Norwood, USA) and expressed as CFU/mL. The tolerance to acidic conditions was determined by comparing the viable cell count after exposure to pH 3 to that at pH 7.

Bile tolerance

The capacity to withstand bile exposure was assessed using a published method [31] with modifications. LAB were harvested by centrifugation of 1 mL of an overnight culture at 6000 ×g for 3 minutes. The pellets were suspended in 1 mL of fresh MRS broth with or without 0.3% (w/v) ox gall and incubated aerobically at 37°C for 3 hours. After incubation, tenfold serial dilutions (up to 10^{-6}) of each bacterial isolate were made using PBS and 50 µL of 10^{-3} to 10^{-6} dilution was spread on MRS agar and incubated aerobically at 37°C for 24 hours. Viability was assessed by counting the number of colonies (CFU/mL) on the plates. Bile tolerance was expressed as a measure of the difference in viable cell counts in MRS with and without bile.

Antibiotic susceptibility testing

Susceptibility to commonly used antibiotics is one of the favorable criteria for the evaluation of probiotic suitability. The disc diffusion test for antibiotic susceptibility was done with five commercially important antibiotics--ampicillin, erythromycin, kanamycin, tetracycline and streptomycin (Oxoid, Basingstoke, UK). The assay was based on a published method with slight alterations [32]. Aliquots of 100 µl of overnight culture were uniformly spread on brain heart infusion (BHI) agar (Oxoid, Basingstoke, UK) plates and the plates allowed to dry for half an hour. Discs containing each antibiotic were placed on the agar with sterile forceps. After 24 hours incubation in aerobic conditions, inhibition zones were measured and strain susceptibility was assessed as reported earlier [32].

Hemolytic activity

The strains were tested for hemolytic activity according to a reported procedure by incubating LAB on blood agar containing 5% sheep's blood (v/v) for 48 hours at 37°C. Strains that gave green-hued zones around the colonies (α-hemolysis) or showed no effect

on the blood plates (γ-hemolysis) were considered to be non-hemolytic, while strains displaying clear blood lysis zones around the colonies (β-hemolysis) were interpreted as hemolytic [33].

Test of ability to metabolize lactose

Minimal MRS media (mMRS) agar plates were prepared using the standard recipe but without meat extract and glucose. After autoclaving, sterile filtered 1% lactose and 50 μg/mL bromocresol purple (BCP) were added to the media. Overnight cultures of the shortlisted strains (100 μL) were spread on mMRS agar plates with lactose as the sole carbon source and incubated for 24 hours at 37°C. Lactose metabolism produces lactic acid which turns the BCP yellow.

Statistical analysis

The experiment were done in triplicate, and results are presented as the mean ± standard deviation. Standard deviations were determined with the Microsoft Excel 2019 software Microsoft Corp., Redmond, WA, USA). Experimental data were analyzed with one-way ANOVA using the SPSS 25.0 software (IBM, Boulder, CO, USA). Statistical differences among means were determined by the Tukey HSD at the 95% confidence interval.

Results

Phylogenetic analysis of LAB strains

To find bacterial strains to control MDR and pathogenic bacteria we tested thirty-two strains from our laboratory collecttion that were obtained previously from sundry local dairy sources (Table 1). The results of partial 16S rRNA gene sequencing showed that twenty-three of the strains (72%) belonged to the genus *Lactobacillus* and nine (28%) to other genera. Among the nine, three isolates were *Ent. faecium,* three were *Weissella* species, two were *Streptococcus lutetiensis*

Table 2. Quantification of the antibacterial activity of LAB strains by microdilution method.

Strain code	*P. aeruginosa* ATCC 9027	*S. aureus* N1	*E. coli* SS1	*S. enterica* ser Typhi D1
PL1	8 ± 0.54	6 ± 0.62	6 ± 0.53	6 ± 0.75
PL3	9 ± 0.15	7 ± 0.25	7 ± 0.25	9 ± 0.56
PL4	10 ± 0.71	6 ± 0.56	6 ± 0.95	6 ± 0.25
PL5	8 ± 0.55	6 ± 0.25	5 ± 0.46	7 ± 0.78
PL6	**5 ± 0.23**	**4 ± 0.46**	**4 ± 0.25**	**5 ± 0.53**
PL7	**6 ± 0.25**	**2 ± 0.63**	**3 ± 0.75**	**4 ± 0.25**
PL8	10 ± 0.78	7 ± 0.25	5 ± 0.56	7 ± 0.95
PL9	8 ± 0.15	7 ± 0.25	6 ± 0.66	6 ± 0.53
PL10	9 ± 0.56	6 ± 0.75	5 ± 0.25	6 ± 0.55
PL11	**8 ± 0.16**	**2 ± 0.54**	**4 ± 0.53**	**5 ± 0.71**
PL12	8 ± 0.75	6 ± 0.53	7 ± 0.73	6 ± 0.62
PL13	**5 ± 0.73**	**2 ± 0.65**	**3 ± 0.27**	**4 ± 0.56**
PL14	12 ± 0.28	7 ± 0.25	6 ± 0.52	6 ± 0.25
PL15	8 ± 0.17	7 ± 0.52	6 ± 0.29	7 ± 0.64
PL16	11 ± 0.57	8 ± 0.25	7 ± 0.78	7 ± 0.73
PL17	12 ± 0.75	7 ± 095	7 ± 0.68	7 ± 0.25
PL18	10 ± 0.51	7 ± 0.75	7 ± 0.25	6 ± 0.33
PL19	12 ± 0.18	7 ± 0.46	7 ± 0.82	7 ± 0.54
PL20	12 ± 0.25	8 ± 0.25	5 ± 0.51	7 ± 0.25
PL21	8 ± 0.45	8 ± 0.75	6 ± 0.86	7 ± 0.62
PL22	10 ± 0.59	8 ± 0.55	7 ± 0.25	8 ± 0.25
PL23	8 ± 0.25	8 ± 0.25	8 ± 0.46	8 ± 0.42
PL24	8 ± 0.82	8 ± 0.96	7 ± 0.59	8 ± 0.75
PL25	10 ± 0.65	8 ± 0.25	7 ± 0.25	8 ± 0.64
PL26	8 ± 0.22	8 ± 0.62	7 ± 0.75	8 ± 0.74
PL27	8 ± 0.71	8 ± 0.75	7 ± 0.51	8 ± 0.25
PL28	8 ± 0.52	8 ± 0.46	7 ± 0.63	7 ± 0.62
PL29	10 ± 0.25	10 ± 0.5	6 ± 0.52	7 ± 0.15
PL30	8 ± 0.47	8 ± 0.25	7 ± 0.75	7 ± 0.35
PL31	10 ± 0.25	8 ± 0.78	7 ± 0.56	7 ± 0.65
PL32	10 ± 0.56	8 ± 0.75	7 ± 0.62	6 ± 0.42
PL33	8 ± 0.75	8 ± 0.52	7 ± 0.51	8 ± 0.52

Values given are minimum inhibitory percentages (MIP) of CFS of LAB strains against various MDR pathogenic bacteria**.** Data are the mean ± SD of at least three independent experiments.

Table 3. Survival of *W. confusa* PL6, *Ent. faecium* PL7, *L. delbrueckii* PL11 and PL13 strains in the presence of low pH and bile. Means within a row are not statistically significant ($p > 0.05$).

LAB Strains	pH assay			Bile assay		
	Cell viability (log CFU/mL)		Reduction in cell viability (log units)	Cell viability (log CFU/mL)		Reduction in cell viability (log units)
	pH 7	pH 3		Bile 0%	Bile 0.3%	
PL-6	10.59±0.15	8.18±0.17	2.41	9.10±0.12	8.59±0.16	0.51
PL-7	8.94±0.13	7.03±0.15	1.91	7.60±0.18	6.08±0.19	1.52
PL-11	8.66±0.09	8.55±0.12	0.11	8.03±0.17	6.79±0.15	1.24
PL-13	7.79±0.25	6.81±0.22	0.98	7.34±0.15	5.79±0.12	1.55

and one was *Leuconostoc lactis.* The species verification was further strengthened by phylogenetic analysis in which the strains clustered in the identified species clades, including twelve species sequences of the NCBI GenBank (Supplementary Figure 1). All the sequences were already deposited in NCBI-GenBank along with location and source of the strains.

Antibacterial activity of LAB isolates

Cell-free supernatant (CFS) from the LAB isolates were tested against the indicator strains using the broth microdilution method and all significantly inhibited pathogen growth. Only four LAB isolates (*W. confusa* PL6, *Ent. faecium* PL7, and *L. delbrueckii* PL11 and PL13) showed maximum inhibition below 6 % of CFS for all tested pathogens (Table 2).

Acid tolerance

Tolerance to acid is one of the prerequisites for an effective probiotic. The LAB isolates, *W. confusa* PL6, *Ent. faecium* PL7, *L. delbrueckii* PL11 and PL13, were chosen for acid tolerance analysis because they had the greatest antimicrobial activity. The strains PL7, PL11 and PL13 showed good tolerance to pH 3 during the 3 hours incubation (Table 3), although the colony count was significantly decreased compared to controls at pH 7. The viability of the isolates decreased with decreasing pH and increasing incubation time. The *W. confusa* isolate PL6 showed the largest decrease in CFU/mL from 3.9×10^{10} to 1.5×10^{8} at pH 3 for 3 hours of incubation, whereas PL7, PL11 and PL13 showed less reduction in the viable cell numbers.

Bile Tolerance

The sensitivity of lactobacilli to oxbile did not exhibit significant variance among the test strains, since all of them tolerated 0.3% oxbile. Out of the four shortlisted strains, PL7 demonstrated the most tolerance albeit slightly as it maintained a log 6 count of viable cells post-exposure to bile for 3 hours (Table 3).

Antibiotic susceptibility

The antibiotic resistance of *W. confusa* PL6, *Ent. faecium* PL7, and *L. delbrueckii* PL11 and PL13 was analyzed (Table 4**)**. All isolates were susceptible to all tested antibiotics; therefore, there is no risk of transmission of antibiotic resistance from the isolates to pathogenic bacteria.

Antibacterial activity of CFSs subjected to heat, acid neutralization and protease digestion

The spectrum of antibacterial activity of select LAB strains was broad as they inhibited the growth of both gram positive and gram negative pathogenic bacterial strains on solid media (Supplementary Table 1). To determine the nature of the inhibitory substances secreted by the four best LAB strains, their CFSs were treated in various ways and then tested for antibacterial activity against pathogens. Proteinase K, trypsin and heat treatment had no impact on the inhibitory effect (*data not shown*). In order to quantifiy and assess the magnitude of the antibacterial activity of elaborated organic acids and metabolities, Cell-free supernatant (CFS) from the LAB isolates were tested against the indicator strains using the broth microdilution method

Table 4. Antibiotic susceptibility of *W. confusa* PL6, *Ent. faecium* PL7, *L. delbrueckii* PL11 and PL13 analyzed using the agar disc diffusion method. (Mean diameters of lytic zones against antibiotics ± SD in mm).

LAB strains	Kanamycin (500 µg/disk)	Tetracycline (80 µg/disk)	Streptomycin (500 µg/disk)	Erythromycin (15 µg/disk)	Ampicillin (10 µg/disk)
PL-6	14±0.12 [S]	27±0.32 [S]	20±0.21 [S]	25±0.23 [S]	37±0.42 [S]
PL-7	15±0.24 [S]	27±0.29 [S]	22±0.23 [S]	22±0.32 [S]	35±0.28 [S]
PL-11	17±0.19 [S]	25±0.22 [S]	20±0.18 [S]	28±0.26 [S]	32±0.32 [S]
PL-13	16±0.09 [S]	25±0.26 [S]	19±0.19 [S]	25±0.07 [S]	29±0.38 [S]

[S] susceptible.

and all significantly inhibited pathogen growth (Figure 2). The CFS of four LAB isolates (*W. confusa* PL6, *Ent. faecium* PL7, and *L. delbrueckii* PL11 and PL13) out of the tested panel was found to be most inhibitory (MIP < 6 % v/v of CFS) for all tested pathogens (Table 2).

pH decrease with grooth of LAB strains

The four LAB isolates were grown on MRS media and decrease in pH was monitored. The pH of the growth media dropped from pH 6.2 to 4.3 following 24 hours incubation, and no statistical difference was observed between the different LAB strains tested (Figure 1). The pH decreased until the stationary phase has been achieved, and after that, it remains more or less constant. The CFS of cultures incubated for 14 hours were used for HPLC analysis.

Organic acids

The organic acid profiles of *W. confusa* PL6, *Ent. faecium* PL7, *L. delbrueckii* PL11 and PL13 are presented in (Figure 3). Lactic acid was the most abundant organic acids produced followed by acetic acid and propionic acid, respectively. The amount of lactic acid production varied among the strains ranging from 1.65 to 2.77 g/L. The highest concentration of

Figure 3. Chromatogram: A Standard organic acids solution, B CFS of the LAB strains.

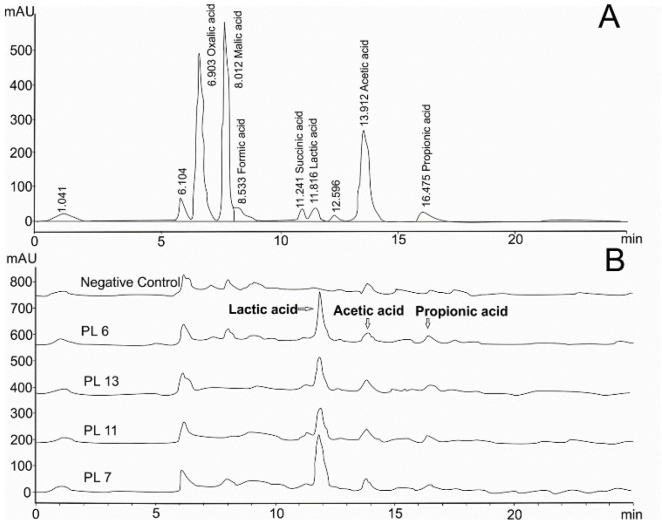
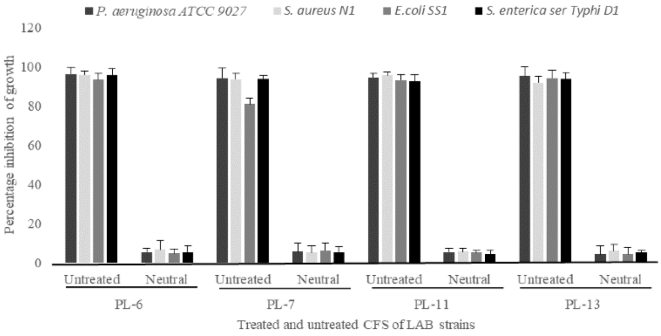


Figure 2. Antagonism of pathogenic bacteria following their exposure to CFS (untreated) and neutralized (Neutral) of select LAB strains. Data are the mean ± SD of at least three independent experiments.



lactic acid (2.77 g/L) was produced by *Ent. faecium* PL7 (Table 5). The acetic acid concentration also varied and was most abundantly produced by *L. delbrueckii* PL13. Production of propionic acid was similar in all four tested strains. Other acids such as oxalic, formic, malic and succinic were produced in trace amounts or not produced by the strains.

Discussion

This study aimed to screen LAB isolated from indigenous dairy sources for those possessing antibacterial activity against MDR and pathogenic bacteria. Fermented dairy products produced locally are of microbiological interest since products such as dahi (yogurt) constitute an integral part of the South Asian diet and are made by the traditional back-slopping method without the use of commercial starters. This approach practiced over centuries has resulted in the creation of a rich blend of mixed cultures containing indigenous species and strain variations differing from region to region [34]. Artisanal dairy products are known to harbor diverse probiotics LAB [35]. Exploring the antagonistic potential of LAB species from indigenous South Asian fermented produce against human pathogens is not without precedent, as many studies suggest [36]; however, their postbiotic characteristics have been little explored even though some have found application in animal feed and cancer therapy [37]. Species identification was made through the widely established 16S rRNA gene sequencing

Table 5. Organic acid quantification by HPLC.

LAB Strains	Lactic acid (g/L)	Acetic acid (g/L)	Propionic acid (g/L)
Enterococcus faecium PL7	2.776 ± 0.02	0.906 ± 0.02	0.599 ± 0.01
Lactobacillus delbrueckii PL11	1.621 ± 0.06	0.979 ± 0.02	0.525 ± 0.01
Lactobacillus delbrueckii PL13	1.653 ± 0.05	0.983 ± 0.01	0.534 ± 0.01
Weissella confusa PL6	2.587 ± 0.03	0.742 ± 0.01	0.579 ± 0.01

technique [38] which revealed all as LAB species. A prevalence of Lactobacillales especially lactobacilli in artisanal dairy fermentations is known [39]. Naturally fermented products of tropical origin have a predominance of mesophilic lactobacillus species such as *L. fermentum* [40] whereas *L. delbrueckii* is a thermophilic starter species, typically found in cheese and fermented milk products [41]. A multitude of antimicrobial substances is known to be elaborated by LAB and many have been identified and characterized for their antimicrobial activity. They range from organic acids derived from primary bacterial metabolism to complex macromolecules arising from bioconversion or protein synthesis [42]. Analysis of CFS represents a reliable way of assessing early on, the potential of candidate probiotics [43]. The characterization of the CFS of these strains which showed an effect of pH neutralization but not of proteolytic enzymes (*data not shown*), and an absence of biosurfactants, suggests that the metabolic acids are the underlying cause of antimicrobial action. Although CFSs of bacterial cultures contain many different metabolites, the bulk of evidence points to organic acids as the principal antibacterial agents [44,45]. Homofermenters such as enterococci can produce large amounts of lactic acid as shown here; consequently, some *Ent. faecium* strains are being used for its industrial production [46]. The *L. delbrueckii*, strains PL11 and PL13, are also obligate homofermenters, producing abundant lactic acid. High lactate production is a hallmark of this species. Of the two species most frequently used as starters in commercial yogurt making, *L. delbrueckii* produces greater amounts of lactate than *S. thermophilus* [47]. Production of both lactic and acetic acids as seen here, can act synergistically to produce low pH [48]. Similar amounts of propionic acid were produced by all four tested strains but at much lower levels than lactic and acetic acids. Propionic acid is not considered a major organic acid of LAB species and consequently does not contribute much to the antibacterial activity against food pathogens compared to lactic and acetic acids [1]. The three organic acids elaborated by these strains are volatile short-chain fatty acids (VSFAs) [49], and of the three, lactic acid (pKa of 3.08) has the weakest antagonistic potential [50]. Lactic acid has a broad spectrum of activity, acting on both Gram-positive and -negative bacteria, as evident in our findings as well as the literature; but, it has been suggested that its inhibitory action is more pronounced on Gram-negative cells owing to cell wall differences [51,52], which was not supported by our findings. This variance in antimicrobial activity observed in this study might be due to subtle differences in the CFS composition of these different species, as organic acids have been reported to activate metabolites by acidification or mediate their insertion into membranes [53]. Even though, the alkaline environment of the colon should neutralize the organic acids generated by the resident LAB cells, a localized pH gradient is widely accepted as a plausible mechanism for pathogen elimination [53].

Acid and bile resistance are prerequisites for probiotic function because ingested strains need to survive the harsh environment of the GIT. These characteristics are often assessed *in vitro* in the preliminary selection of a probiotic strain. The human stomach typically secretes about 2.5 L of gastric juice daily which has a pH varying from a low of 1.5 (fasting) to 3.5, after a meal has been eaten [54]. About 1 L of bile synthesized in the liver is secreted into the small intestine daily, which an ingested potential probiotic also needs to survive [55]. The ability of these LABs to tolerate bile salts at a concentration of 0.3% is physiologically significant as being similar to the bile concentration in the human intestine [56]. Losing up to a third of cells as seen here for the *W. confusa* strain following exposure to physiological levels of acid and bile should not be considered as grounds for precluding it as a potential probiotic [57]. The tolerance of bacteria to such a harsh environment produced by acid and bile is attributed to the production of ATPases, bile salt hydrolases and other enzymes [58] and our results suggest that these strains are likely to persist in the human GIT.

Although, LABs are extensively used in the production of fermented food products and food preservation, these microorganisms have the potential ability to transfer antibiotic resistance to pathogenic species. Therefore, evaluation of antibiotic susceptibility is strongly advised for all bacteria with application in the food industry, especially those which are to be used as probiotics [59]. Disc diffusion assay is a CLSI recognized way of evaluating antimicrobial sensitivities [60]. A susceptibility towards most of the commonly used antibiotics, therefore, obviates concerns of antibiotic resistance transmission from putative probiotics to other pathogens or gut commensals. Hemolytic activity is a trait associated with virulence in some food-associated microorganisms. Our results concur with past findings where LAB strains of dairy origin were reported to have no hemolytic activity [61].

A functionally active bacterial β-D-galactosidase found in many LAB is involved in lactose breakdown.

There is evidence of probiotics having an ameliorative effect on lactose intolerance [18]. Exogenous intake of lactases of microbial origin has shown promise as a treatment option for lactose intolerance [62].

Conclusions

W. confusa PL6, *Ent. faecium* PL7, and *L. delbrueckii* PL11 and PL13 were all capable of fermenting lactose and producing lactic acids that inhibited MDR human pathogens *in vitro*. The variation in the quantities of organic acids produced in our LAB strains is in line with previous contentions where type and quantities of post-biotics of lactobacilli vary from strain to strain [21]. Post-biotics are purported to not only enhance the potency of probiotics but also present a more effective and safer way of delivering desired health effects without the technical challenges associated with probiotics. Their value in food manufacturing and preservation cannot be overemphasized especially in the context of developing countries [63]. The quantities of lactic acid produced by the *L. delbrueckii* species in our study are similar to what has been reported elsewhere [64]. In contrast, the high levels of lactic acid observed in the *W. confusa* strain, a typically facultative heterofermenting species which matched and even surpassed that of some the homofermentative lactobacillus strains in our work suggests its post-biotic value. However, unlike *Ent. faecium* which because of its distinct probiotic applications is a good candidate for qualified presumption of safety (QPS) status [65], the investigations of *W. confusa* carried out so far, suggest that its potential as a probiotic and as a post-biotic must be empirically demonstrated at the strain level [66] before it could be earmarked for the probiotic designation.

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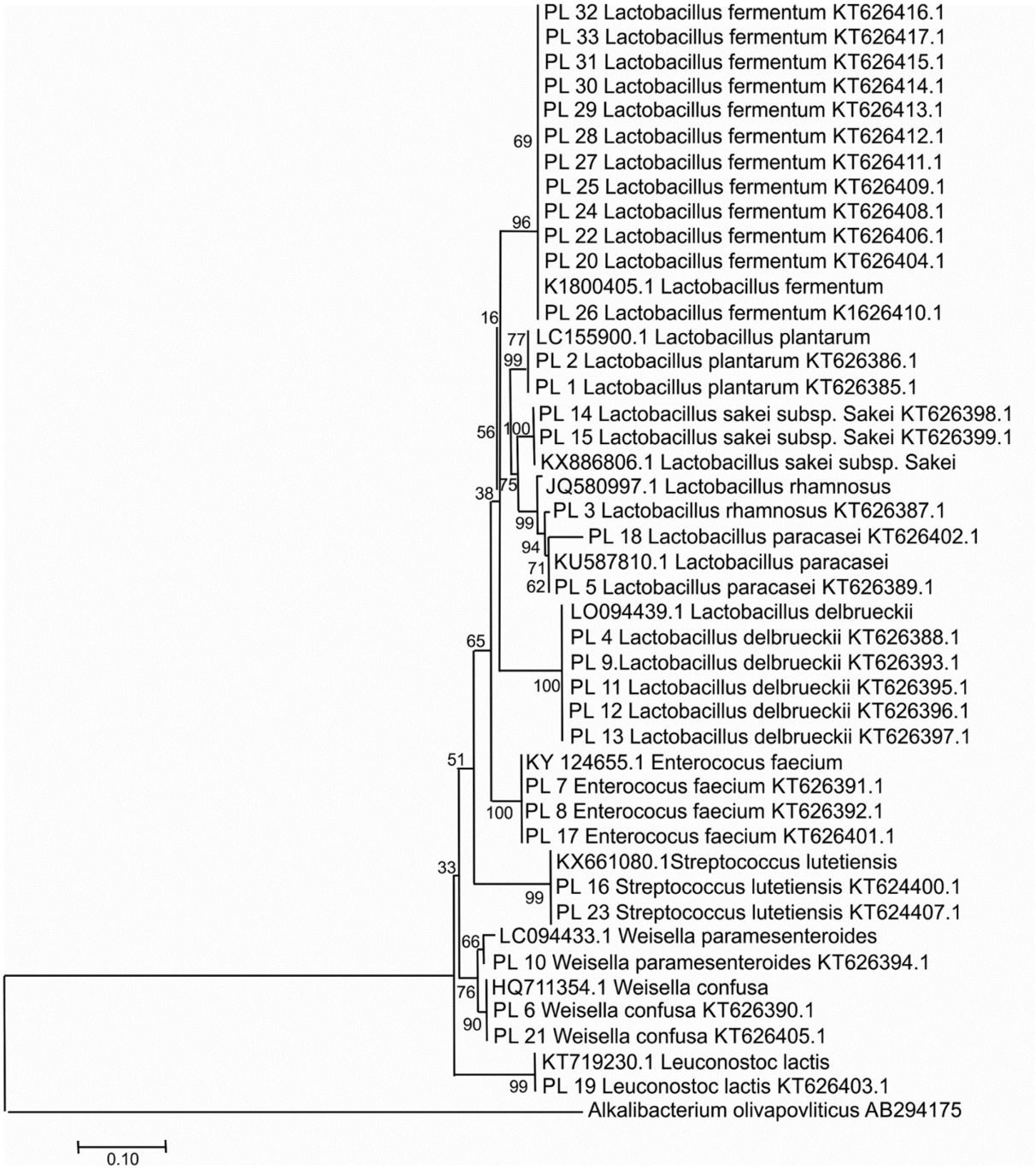
Annex – Supplementary Items

Supplementary Table 1. Antibacterial activity of the shortlisted LAB strains by agar well diffusion assay (mean ± SD of diameter of inhibition zones in mm).

Indicator strains	PL6	PL7	PL11	PL13
E. coli SS1	17.27 ± 0.39	18.37 ± 0.28	19.92 ± 0.31	18.43 ± 0.34
P. aeruginosa ATCC 9027	18.26 ± 0.38	19.96 ± 0.35	17.23 ± 0.23	19.73 ± 0.32
S. enterica ser Typhi D1	19.25 ± 0.50	20.37 ± 0.49	21.13 ± 0.38	20.62 ± 0.25
S. aureus N1	19.25 ± 0.44	19.87 ± 0.55	19.75 ± 0.45	19.75 ± 0.28

Supplementary Figure 1. Phylogenetic analysis of LAB isolates.



This was inferred using the neighbor-joining method by MEGA7. NCBI Accession numbers are also given with the LAB strains.